

Warren Strober
Naoki Asano
Ivan Fuss
Atsushi Kitani
Tomohiro Watanabe

Cellular and molecular mechanisms underlying NOD2 risk-associated polymorphisms in Crohn's disease

Authors' addresses

Warren Strober¹, Naoki Asano², Ivan Fuss¹, Atsushi Kitani¹, Tomohiro Watanabe³

¹Mucosal Immunity Section, LHD, NIAID, NIH, Bethesda, MD, USA.

²Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai Miyagi, Japan.

³Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan.

Correspondence to:

Warren Strober

Mucosal Immunity Section, LHD, NIAID

National Institutes of Health

Building 10-CRC, Room 5-3940

10 Center Drive

Bethesda, MD 20892-1456, USA

Tel.: +1 301 496 6810

Fax: +1 301 402 2240

e-mail: Wstrober@niaid.nih.gov

Acknowledgements

None of the authors have a conflict of interest.

This article is part of a series of reviews covering Mucosal Immunity appearing in Volume 260 of *Immunological Reviews*.

Summary: The discovery that polymorphisms in the NOD2 (nucleotide-binding oligomerization domain containing 2) gene are associated with a greatly increased risk for the development of Crohn's disease has provided a means to achieve a deeper understanding of the dysregulation of mucosal immune responses to the commensal intestinal organisms that is thought to underlie this disease. NOD2 is a NOD-like receptor (NLR) family member that senses and responds to bacterial wall peptides; thus, the most widely held view of the relation of the NOD2 polymorphisms with Crohn's disease is that these polymorphisms lead to deficient immune responses to gut bacteria, and these, in turn, lead to quantitative or qualitative changes in the bacterial population in the gut lumen or lamina propria that cause inflammation at this site. Initially, this view was based mainly on the observation that defective NOD2 function can result in reduced α -defensin production by intestinal Paneth cells and that such impairment leads to loss of host defense against gut bacteria. In this review, we reconsider this possibility and marshal evidence that it is not in fact likely to be a prime element of Crohn's disease causation. More recently, evidence has been accumulating that the NOD2 dysfunction leads to Crohn's inflammation by inducing changes in the gut microbiome that influence immune effector or regulatory function. We review the strengths and weaknesses of this emerging hypothesis. Finally, we consider the possibility that NOD2 dysfunction can lead to inflammation because of a second and somewhat overlooked aspect of its function, that as an immunoregulator of innate immune responses. In particular, we review the body of evidence that NOD2 stimulation activates a cross-tolerance response that downregulates and thus prevents excessive TLR responses that cause Crohn's inflammation.

Keywords: NOD2, Crohn's disease, defensins, colitis, microbiome, IRF-4

Introduction

In the last decade, genome-wide association studies have uncovered over 150 genes that have coding or non-coding polymorphisms occurring at a higher frequency in patients with Crohn's disease (CD) than controls and that therefore are assumed to be contributing to its pathogenesis (1). By far, the most striking of these polymorphisms are those in

Immunological Reviews 2014

Vol. 260: 249–260

Printed in Singapore. All rights reserved

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons

Ltd

Immunological Reviews

0105-2896

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Immunological Reviews 260/2014

the coding region of the nucleotide-binding oligomerization domain 2 (NOD2/CARD15) gene, a set of polymorphisms that confer a 17.1-fold increased risk for CD development when present in homozygous or compound heterozygous form. NOD2 is a member of the NLR family of innate immune recognition factors that senses muramyl dipeptide (MDP), a peptide derived from the peptidoglycan component of the bacterial wall (2). The major polymorphisms of NOD2 associated with CD are located in the leucine-rich repeat (LRR) region of the molecule and decrease its ability to interact with MDP; they thus represent loss-of-function genetic abnormalities (2, 3). Given the above relation of NOD2 to bacterial peptide recognition, disturbances in NOD2 function are well positioned to play a role in facilitating the excessive response to microflora comprising the gut microbiome thought to underlie this disease. The question is how.

NOD2 function and defensin production

The earliest and still the dominant theory of NOD2-associated risk for CD came from the observation that NOD2 is expressed not only in antigen-presenting cells (macrophages and dendritic cells) in the lamina propria tissue underlying epithelial cells, but also in intestinal epithelial cells (Paneth cells mainly) (4). This, plus the fact that NOD2 stimulation is necessary for the production of intestinal cryptdins (defensins), relatively low molecular weight antibacterial proteins secreted by Paneth cells that are located at the base of the crypts of the small intestine or the ascending colon (5, 6), gave rise to the possibility that loss of NOD2 function led to decreased cryptdin production and thus changes in the gut microbiome that trigger Crohn's inflammation (6). In this formulation, CD is a subtle type of immunodeficiency that fosters the unfettered expansion of intestinal organisms (or products of these organisms) with otherwise low or absent pathogenicity. This, in turn, overwhelms normal epithelial cell barrier function and causes inflammation functionally equivalent to that observed in models of colitis characterized by actual loss of epithelial barrier function such as dextran sodium sulfate (DSS)-colitis or by genetically engineered loss of epithelial integrity due to expression of dominant negative N-cadherin (7).

Several kinds of evidence have been obtained to support this view. The earliest came from some of the initial studies of NOD2-deficient mice by Kobayashi et al. (6) that showed that such mice were subject to more severe systemic infection following oral challenge with *Listeria monocytogenes*;

in addition, these authors showed that isolated Paneth cells obtained from the NOD2-deficient mouse small intestine exhibited reduced cryptdin production. Later studies that tended to corroborate these data showed that isolated crypts from NOD2-deficient mice had reduced capacity to kill several intestinal pathogens, not only *L. monocytogenes*, and indeed such mice exhibited reduced resistance to infection with these or other pathogens, particularly those infecting the gastrointestinal tract (8, 9). In addition, NOD2-deficient mice exhibited increased numbers of commensal organisms in the lumen of their terminal ilea and changes in the composition of this microbiome (10) (see discussion below). Finally, in one particularly striking study it was shown that following challenge with *Helicobacter hepaticus*, NOD2-deficient mice as compared with wildtype (WT) mice exhibited increased expansion of this organism in the terminal ileum and developed granulomatous inflammation limited to this area of the gut characterized by a strong Th1 cytokine response (10). In further studies, it was shown that transfer of WT bone marrow into NOD2-negative recipients did not protect recipients from the development of this inflammation, whereas transfer of NOD2-negative bone marrow into WT recipients maintained protection; furthermore, NOD2-negative mice expressing a human α -defensin 5 transgene expressed only in Paneth cells were protected. This study thus established that NOD2 expressed in epithelial cells (i.e. Paneth cells) clearly functions as a host defense factor and that this host defense is most likely expressed through the production of α -defensin. Nevertheless, this study and related studies fell short of establishing that NOD2 dysfunction manifesting as decreased Paneth cell α -defensin production was the basis of increased risk for CD associated with NOD2 polymorphisms, because they did not directly provide data relating to α -defensin production in CD patients and thus did not tie decreased α -defensin production to defective handling of commensal organisms normally populating the human gut.

To obtain evidence addressing this latter issue, α -defensin expression was assessed in patients with CD with or without a NOD2 risk polymorphism. In initial studies, it was shown that those CD patients with ileal inflammation and a NOD2 polymorphism had significantly decreased Paneth cell α -defensin 5 as well as non-significant decrease in α -defensin 6 mRNA production as compared with those with ileal inflammation without a risk polymorphism (11, 12). This decrease was specific in that mRNA levels of other Paneth cell products were not decreased. However, the α -defensin

decrease was limited to patients with only one of the three known risk polymorphisms [the frameshift (Leu1007fsins-Cys) polymorphism associated with the greatest loss of NOD2 function] and was not observed in mRNA from inflamed colon; in addition, the significance of the reduction was somewhat mitigated by the fact that patients with ileal disease without a NOD2 risk polymorphism also displayed a major reduction in α -defensin production compared with healthy controls, albeit not as great as that exhibited by patients with a NOD2 polymorphism. This observation suggested that the decrease in α -defensin production was largely due to the underlying inflammation in spite of data in one study showing that patients with different levels of inflammation by histologic criteria had similar levels of decrease (12). More importantly, it suggested that the decreased α -defensin production was not a primary disease factor and that decreased NOD2 expression does not necessarily equate with decreased α -defensin expression.

These latter conclusions were in fact supported by a second and independent study of α -defensin production in a larger group of ileal CD patients. Here, it was found that reductions in α -defensin production in patients with and without polymorphisms were directly proportional to the extent of inflammation and the residual Paneth cells as measured both histologically and by expression of a Paneth cell marker, PLA2G2A and a marker of epithelial cell mass, VIL1 (13). In addition, patients with NOD2 polymorphisms, but without ileal inflammation (i.e. patients not included in the first study), exhibited normal α -defensin levels. These data thus reinforced the idea that decreased α -defensin production in CD with associated risk NOD2 polymorphisms is a secondary effect of the underlying inflammation rather than on the NOD2 polymorphism and, furthermore, suggested that the findings in the original study of α -defensin production in patients with polymorphisms arose from the fact that these patients had more severe disease for reasons other than α -defensin production.

The latter conclusion leaves open the question of why patients with defective NOD2 function do not have a primary defensin production abnormality, given data on the relation of NOD2 to defensin production derived from mouse studies. A possible answer to this question comes from a recent study in which NOD2 deficiency in mice was correlated with Paneth cell α -defensin production under carefully controlled conditions (14). In this study, α -defensin 4 and α -defensin-related sequence 10, both Paneth cell derived cryptdins, were assessed in NOD2-deficient and littermate co-housed WT control C57BL/6 mice derived from the same founder mice.

Thus, this study differed from previous studies wherein mice with differences in their genetic and/or environmental backgrounds were compared. In this instance, no differences in production of these cryptdins at either the mRNA or protein level was found and the mice also exhibited equal cryptdin antimicrobial activity; in addition, production of other Paneth cell antimicrobial peptides was equivalent (with a minor exception). Finally, the NOD2-deficient and WT control (co-housed) mice exhibited minimal differences in fecal microbial composition. These surprising observations strongly suggest that in co-housed C57BL/6 mice, normal levels of defensin production can be maintained in spite of NOD2 status and thus the latter has little effect on the intestinal microflora, at least in the absence of pre-existing intestinal inflammation. In addition, they hint that α -defensin production by Paneth cells is a complex process that is not entirely under the control of NOD2. This latter point opens the door to the possibility that α -defensin production in humans with or without intestinal inflammation can occur in the presence of NOD2 dysfunction.

From the above discussion, one might conclude that the initial study of NOD2 deficiency in which susceptibility to *L. monocytogenes* infection or indeed other studies of NOD2 deficiency and GI infection were explained by α -defensin production abnormalities are misleading as, in reality, these abnormalities may have been due to differences in the genetic and environment backgrounds of the mice being compared that were independent of NOD2 status. However, such differences do not fully account for the observation that *H. hepaticus* infection of NOD2-deficient mice leads to granulomatous inflammation unless 'rescued' by transgenic expression of a human defensin gene in epithelial cells. This follows from the fact that even if NOD2 deficiency leads to only a partial decrease in α -defensin production (at least in some mouse strains) this study showed that increased α -defensin production in Paneth cells was protective. On the basis of the latter finding as well as observations to be described below, it is likely that NOD2-deficient mice do in fact manifest decreased α -defensin production and this is the basis of decreased host defense function with respect to certain pathogens. However, one still cannot conclude from this that NOD2 risk polymorphisms in CD patients act primarily through their effect on α -defensin production in view of the fact that no defects are observed in such production in CD patients with a risk polymorphism without disease of the terminal ileum and the fact that there is considerable reason to believe that decreased α -defensin production in CD patients reflects the presence of inflammation.

Paneth cell function and NOD2

Further insights into the role of NOD2 as a necessary factor for defensin production come from a more general consideration of Paneth cell function, i.e. the cellular origin of the bulk of epithelial NOD2 production.

For some time now, it has been known that mice with total ablation of Paneth cells (in the absence of other epithelial cell defects), i.e. mice bearing a diphtheria toxin A transgene in the cryptdin-2 locus, do not develop small intestinal inflammation and are free of bacteria within the intestinal crypts (15). On the other hand, it has been shown that mice deficient in matrix metalloproteinase 7 (MMP7 KO mice), i.e. mice deficient in an enzyme necessary for activation/secretion of defensins in Paneth cells and therefore lacking functional defensin production at this site, are more susceptible to infection by pathogens and, perhaps more importantly to the present discussion of NOD2, exhibit major shifts in the composition of their bacterial microflora while maintaining normal total numbers of small intestinal bacteria (16). Interestingly, these microflora shifts are characterized by increases in *Firmacute* bacteria (especially *Clostridia*) and decreases in *Bacteroidete* bacteria, bacterial species that also exhibit changes in numbers in patients with CD. One caveat to the acceptance of these data is that the MMP7 KO mice and their littermate controls were not co-housed; it is therefore possible that shifts in microbial composition might disappear in studies of deficient mice and control mice reared under identical conditions were compared, as in the study mentioned above. It should also be mentioned that the effect of the shift in the microbial population was not pursued in the above studies so we have little or no insight into the possible consequences of the shift. As the mice with deficient defensin production exhibited higher levels of *Clostridia*, bacteria that have been shown to mediate suppression of colonic inflammation (see further discussion below), it is possible that the shift actually protected the mice from inflammation.

Other studies of the relation of TLR signaling with Paneth cell function cast further light on the latter's effect on intestinal microflora. Thus, it has been shown that whereas NOD2 affects defensin production by Paneth cells, its absence leads to increased production of other antimicrobial peptides including RegIII β , RegIII γ , and RELM β and the latter factors are under the control of TLR (MyD88) signaling (5). Moreover, Paneth cells that lack the ability to undergo TLR stimulation by enteric bacteria because of MyD88 deficiency exhibit increased penetration of commensal bacteria

into the lamina propria and mesenteric lymph nodes as well as increased penetration and widespread dissemination of pathogenic bacteria. Finally, ablation of Paneth cells by a diphtheria toxin strategy similar to that mentioned above leads to increased penetration of commensal and pathogenic bacteria. As MyD88-deficient mice still maintain NOD2 signaling, these results indicate that NOD2 alone cannot limit bacterial penetration and, moreover, that TLR signaling also has an important role in this process. Whether or not these results thus assign NOD2 an unnecessary role in the initiation of the bacterial penetration essential to the development of CD-like inflammation remains to be explored in further studies.

Additional insights into Paneth cell function that are relevant to NOD2 and the latter's effect on the bacterial microbiome are recent studies showing that *Toxoplasma gondii* infection induces a Th1 (IFN- γ), response that is responsible for Paneth cell death and the appearance of an altered microflora containing an increased number of Enterobacteriaceae (17). These data show that Th1 cell inflammation has a detrimental effect on Paneth cells, which could explain the correlation between the severity of inflammation and the decrease in α -defensin production found in CD discussed above. In addition, they show that global loss of Paneth cell function due to loss of Paneth cells can also affect the composition of the intestinal microflora in the presence of an inflammation of infectious origin originating independently of the Paneth cell dysfunction. Logically, this applies to non-infectious inflammation as well, such as that in CD.

NOD2 deficiency and the composition of the intestinal microflora

Given that total loss of defensin production in mice with MMP7 deficiency results in shifts in the composition of the intestinal microbiome, it is possible that NOD2 deficiency has the same effect. Evidence that this is in fact the case comes from several recent studies, although the results have to be considered tentative because the NOD2-deficient and control groups were not co-housed and thus the results may have arisen from an environmental discrepancy. In two of these studies, NOD2-deficient mice exhibited a larger ileal (mucosa-attached) population of bacteria comprising both the *Bacteroidete* and *Firmacute* phyla than did control mice and in one of these studies, these changes were noted quite early in life (18, 19). In a third study, an increase in *Bacteroidetes* was again found, but in this case, it was not associated with a change in *Firmacutes* and was associated with a decrease in

Proteobacteria (8). Thus, while in these studies NOD2 deficiency was reproducibly associated with changes in the ileal microflora, these changes were somewhat inconsistent.

As in the case of the studies of Paneth cell function, a question that immediately arises concerning the changes in the intestinal microflora associated with NOD2 deficiency is whether or not these changes increase susceptibility to colitis. At first glance, the question would be answered negatively as, as briefly mentioned above, the bacterial subtypes associated with the *Firmacutes* and the *Bacteroidetes* have been shown to contain species that induce suppressor cells with anti-inflammatory properties. This negative answer would, in addition, be supported by one study in which it was found that NOD2-deficient mice do not exhibit more severe DSS-colitis than normal counterparts, although there was evidence of greater tissue penetration of bacteria in the deficient mice (20). However, in another study, it was found that WT mice co-housed with either NOD2-deficient or RIP2-deficient mice exhibited more severe DSS-colitis than non-cohoused WT mice (21). The more severe disease was associated with higher IL-6 production and increased expression of claudin 5; in addition, it was associated by a greater tendency to develop tumors induced by injection of azoxymethane (AOM) at the time of DSS administration. Finally, the increased colitis was ameliorated by treatment of mice with antibiotics and the increased colitis/tumorigenesis could be communicated to germ-free mice recolonized by fecal transplants from NOD2-deficient mice. These studies provided rather strong evidence that NOD2 deficiency does, in fact, result in a colitis-enhancing microflora that can aggravate a pre-existing colitis-inducing condition, in this case DSS-colitis. Such microflora, however, fall short of being a true colitogenic microflora that can cause disease in a normal, unperturbed mouse, such as the microflora developing in so-called TRUC mice (RAG2-deficient and T-bet double-deficient mice) that develop a colitis associated with a microflora that transmits disease to co-housed normal mice (22, 23).

Analysis of the bacterial microflora in recipients of colitis-enhancing fecal flora versus recipients of non-colitis-enhancing flora (based on 16s rRNA technology) revealed shifts in bacterial sub-types, most notably an increase in *Bacteroidetes* and a decrease in *Firmacutes*. However, the genera within these phyla comprising these changes were not among those shown previously to have effects on immune regulation. In addition, these changes were not closely consonant with changes in intestinal tissue- or ileal tissue-associated bacterial communities noted in patients with CD and risk-associated NOD2 polymorphisms. In one study, it was shown that ileal

bacteria of such patients was characterized by increases in both *Bacteroidetes* and *Firmacutes* and, while the former phylum contained an increase in *Bacteroides*, the latter phylum contained a decrease in *Faecalibacterium prausnitzii* (18). In a second study, tissue-associated bacteria contained a decrease in *Firmacutes* bacteria, the latter including various *Clostridia* genera as well as *Faecalibacterium* and an increase in *Proteobacteria*, the latter including *Eschericia* genera (24). Thus, it could hardly be said that the colitis-enhancing microflora in NOD2-deficient mice mimics that possibly present in CD patients. Another important fact is that in the second study, the same microflora abnormalities were found in patients with the risk-associated autophagy gene, ATG16L1; thus, this microbial pattern was not specifically associated with NOD2 dysfunction.

Whereas NOD2 deficiency in the studies described above led to more severe DSS-colitis, a different picture obtained with respect to the effect of NOD2 deficiency on the relatively mild experimental colitis observed in TNBS-colitis of C57BL/6 mice; in this case, mice with NOD2 deficiency exhibited decreased colitis compared with control mice raised in a similar environment (25). Extensive analysis of this unexpected result showed that it could be attributed to the fact that NOD2-deficient mice, compared with control mice, develop increased numbers of Foxp3 negative regulatory cells that bear surface TGF- β bound to latency-associated protein (LAP), so-called LAP⁺ cells regulatory cells that do not express Foxp3, but nevertheless do have the capacity to suppress colitis when transferred to recipients challenged with TNBS. In previous studies, such *lamina propria* regulatory cells were found to increase in response to administration of agents that cause decreased epithelial barrier function such as ethanol administration and thus their increase appears to be a homeostatic response of the mucosal immune system to the entry of commensal bacteria into the *lamina propria* that occurs as a result of such decreased barrier function and that might otherwise cause inflammation. In NOD2-deficient mice, they also arise from decreased epithelial barrier function, in this instance, probably caused by the fact that the deficiency is associated with low-level (sub-inflammatory) increases in IFN- γ and/or TNF- α production that affect barrier function through their effect on epithelial tight junctions. Such increased cytokine production is likely caused by increased responsiveness to TLR stimulation consequent to the NOD2 dysfunction (see discussion below) that may then be aggravated by bacteria or bacterial products that secondarily enter the *lamina propria* due to the altered barrier function and then stimulate dendritic cells that induce the regulatory cells. This possibility is favored by the fact that regulatory cells are not

increased in NOD2-deficient mice treated with antibiotic regimens that greatly reduce intestinal bacterial populations; in addition, NOD2-deficient mice contain lamina propria dendritic cells that display an increased capacity to induce LAP⁺ regulatory cells upon TLR2 ligand stimulation. Another important fact with respect to the microflora is that co-housing studies showed that NOD2-deficient mice co-housed with normal mice exhibited increased colitis compared with deficient mice reared alone. This argues that the microbiome of the deficient mice was indeed changed, but that the change led to increased protection from colitis, not decreased protection as in DSS-colitis. Overall, these studies reveal that lack of NOD2 function can paradoxically prevent colitis in situations in which entry of microflora is relatively mild (as in the TNBS-colitis of C57BL/6 mice) because in this situation, a homeostatic regulatory mechanism comes into play that suppresses inflammation. However, when entry of luminal bacteria or their products is massive, as in DSS-colitis or perhaps in CD, this homeostatic mechanism is overwhelmed and NOD2 deficiency now resumes its more usual pro-inflammatory effect.

Changes in the intestinal microflora in inflammatory bowel disease

To put the above description of changes in intestinal microflora associated with NOD2 deficiency into context, it is useful at this point to briefly discuss the panoply of abnormalities in microflora communities found in CD generally, not just those with specific genetic polymorphisms.

The data here are somewhat variegated, reflecting differences in the patient groups studied and in the specific methodology applied to identify bacterial profiles in the patients. The most comprehensive study was conducted by Frank et al. (26) who subjected small and large intestinal tissue of large groups of CD and ulcerative colitis (UC) patients to 16S rRNA analysis. Differences between patients and controls were identified in only about one-third of CD patients and one quarter of UC patients and these consisted mainly of decreases of bacteria in the Firmacute and Bacteroidete phyla; in addition, bacteria in the Proteobacteria phyla was increased in the small intestine of CD patients (26). Patients with these changes were younger and more likely to have abscess formation, a feature of more severe disease. However, there was no evidence that the bacterial population contained an increased population of recognized pathogens.

Other studies of smaller groups of patients provided data showing that, at least in some patients, the Firmacute population decreases included decreases in the *F. prausnitzii* bacteria, i.e. bacteria belonging to the Clostridial group of organisms

that have been shown in one study to induce intestinal regulatory cells; thus, this decrease might be associated with disease because it leads to disturbed mucosal homeostasis (27–34). This possibility also derives support from a recent study of germ-free mice whose intestinal microbiome was replete with bacteria from human feces, 17 strains of Clostridial organisms acting in concert have been identified as the gut bacteria that are specifically able to induce suppressor cells and that administration of these bacteria prevent experimental colitis (35). Moreover, 5 of these 17 strains are reduced in abundance in 20 UC patients relative to 15 healthy controls. While these studies require confirmation in larger, more defined patient populations, at the moment, they suggest that decreased numbers of suppressor cell-inducing bacteria do play a primary or secondary role in inflammatory bowel disease.

A case can also be made that decreases in *Bacteroidetes* bacteria also lead to decreased mucosal regulation, as this bacterial phylum harbors organisms (*Bacteroides fragilis*) that have been shown to induce regulatory cells in mice (36, 37). However, this conclusion is clouded by the finding that in some studies, increased numbers of *Bacteroides* organisms were found, including *B. fragilis* organisms (38). In addition, *Bacteroides* organisms were not among those identified as suppressor cell-inducing bacteria in the above-mentioned studies of Clostridial organisms (35).

The increases in Proteobacteria in the small intestine of Crohn's patients are also of interest as this phylum harbors *E. coli* organisms that may contain a particular sub-population of 'semi-pathogenic' organisms, so-called adherent-invasive *E. coli* (AIEC) that have been proposed as possible inducers of CD inflammation, especially by Darfeuille-Michaud et al. (39). AIEC consist of *E. coli* that have the capacity to adhere to epithelial cells via pili that bind to CEACAM6 on the epithelial surface (40); in addition, they bind to Peyer's patch M cell glycoprotein (GP2) via long polar fimbria and thus have the potential to gain entry to the mucosal lymphoid tissue via the Peyer's patches (41). Finally, AIEC resist degradation when taken up by macrophages and induce the latter and perhaps other cells to produce pro-inflammatory cytokines (42, 43).

These various pathogen-like properties of AIEC have led to the view that these organisms are a primary cause of CD. However, several observations rule against this possibility. First, AIEC are found in only about a quarter of CD patients and then only in the small intestine; in addition, they occur in some 6% of controls and in substantial numbers of CD patients without small bowel disease (39). Second, AIEC do

not appear to enter the *lamina propria* in areas that are not already characterized by ulceration despite widespread epithelial attachment; such entry would be critical for the initiation of a CD-type inflammation. Third, CEACAM6 expression is upregulated by pro-inflammatory cytokines so that AIEC's colonization of the epithelium of the small intestine is likely to be a secondary event occurring after inflammation has already occurred (40). For these and other considerations, it is likely that colonization of the small intestine with AIEC is not a primary driver of Crohn's inflammation, but one which nevertheless has considerable potential to intensify pre-existing disease.

Given the presumption that NOD2 deficiency leads to a colitogenic microbiome, at least in mice, one might speculate that Crohn's patients with NOD2 risk polymorphisms are more susceptible to AIEC colonization. This has not been carefully assessed in that in the one study of such patients in which an increase in *Proteobacteria* was found, it was not determined if these organisms included AIEC (24). Studies of this question in mice with NOD2 deficiency initially treated with antibiotics to allow transient AIEC colonization in this species showed that while NOD2 deficiency was associated with increased persistence of colonization as well as increased passage into mesenteric nodes, the AIEC did not cause colitis. However, such colonization did lead to more severe DSS-colitis (44). These data again suggest that AIEC colonization is not a primary cause of intestinal inflammation, but can aggravate pre-existing inflammation.

The above data relating to changes in the gut microbiome in inflammatory bowel disease (particularly CD) as well as somewhat similar changes encountered in mice with NOD2 deficiency raise a fundamental question as to the role of these changes in disease pathogenesis. The most reasonable answer to this question at this point is that most of the microbiome shifts are driven by the inflammation and the latter's effect on how epithelial cells interact with the overlying bacterial community embedded in the mucus layer in contact with the epithelium. This view of the microbiome changes as secondary effects is supported by the fact that these changes are noted only in a minority of patients and then, for the most part, only in the small intestine. In the case of organisms that have been shown to be decreased in number in some patients with CD such as *Firmacutes* and *Clostridia* organisms, it seems possible that the inflammation creates conditions that cause selective decreases in the survival of particular organisms. This possibility is suggested by the observation that decreased levels of *F. prausnitzii*, the *Clostridia* organism mentioned above that has been shown to mediate anti-inflammatory regulatory

effects, are found to be normalized in response to effective reversal of immunologic abnormalities (33). Whether a similar normalization also applies to other *Clostridia* strains or other organisms that have been shown to have regulatory function remains to be seen. If so, one must consider the possibility that intestinal inflammation arising primarily from an immunologic abnormality can be secondarily intensified by effects of the inflammation on anti-inflammatory regulatory organisms.

In the case of organisms that promote colitis such as the aforementioned AIEC in humans, it has been already mentioned that cytokine-induced CEACAM6 expression resulting from the inflammation creates conditions in the small intestine that favor AIEC colonization. Similar inflammation-associated changes in the intestinal microenvironment may apply to other organisms with the potential to intensify a pre-existing inflammation such as the prevotella organisms in mice with NLRP6-deficiency with DSS-colitis (45). A somewhat different explanation of the ability of colitogenic organisms causing disease in TRUC mice is necessary because in this case, the organisms involved causes *de novo* disease in the uninfamed bowel. Thus, in this case, one has to postulate that the colitogenic organisms have inherent properties that can initiate gut inflammation. This said, even here, the inflamed gut plays a permissive role in that TRUC inflammation is decreased or eliminated by administration of agents that address the inflammation and not the 'infection' (14). Evidently, colitogenic organisms ultimately persist only within an inflamed micro-environment.

NOD2 regulation of gut innate immune responses

The above analysis of the role of NOD2 risk polymorphisms in the pathogenesis of CD raises several questions concerning the notion that the polymorphisms cause disease solely or primarily because they introduce a mucosal immunodeficiency. This, however, should not be construed to imply that NOD2 has no meaningful host defense function. The fact is that the ability of MDP to initiate NOD2 activation followed by induction of NF- κ B and MAPK activation is well documented and while such activation is considerably less robust than that typically elicited by TLR2 or TLR4 ligands, NOD2 co-stimulation of TLR responses leads to impressive elevations in the latter responses (46). In addition, there is evidence that NOD2 (as well as NOD1) binds to ATG16L1, a key factor in the initiation of autophagy, and that such interactions 'target' autophagic machinery to the site of entry of bacterial pathogens at the cell membrane

(47, 48). As uptake and destruction of bacteria by autophagic vesicles have emerged as an important mechanism for the elimination of invading pathogens, such interaction is an additional factor that implicates NOD proteins in host defense. Nevertheless, as already alluded to above, humans with NOD2 dysfunction associated with polymorphisms are not subject to increased infections with known pathogens; thus, the host defense function of NOD2 appears to be circumscribed in humans and, for this reason, poorly capable of explaining how commensal organisms with lower invasive potential than pathogens can be causing CD.

The above uncertainty concerning the importance of NOD2 host defense function in humans in general and CD in particular underscores the relevance and importance of a second major theory of the way NOD2 risk polymorphisms influence the appearance of CD. This relates to increasing evidence that NOD2 risk polymorphisms operate primarily via effects on NOD2 regulation of innate immune mechanisms that lead to over-active TLR responses and inflammation.

The earliest data to uncover this NOD2 function were reported by Watanabe *et al.* (49, 50), who showed that prestimulation of both mouse and human dendritic cells with MDP led to downregulation of subsequent responses to PGN, a TLR2 ligand, as well as to a broad range of other TLR stimulants. This observation was subsequently verified *in vivo* with studies that showed that augmentation of NOD2 signaling in mice by administration of MDP to mice bearing a normal NOD2 transgene was accompanied by the appearance of lamina propria cells displaying reduced TLR responses, whereas such administration to mice bearing a NOD2 transgene with the frame-shift polymorphism found in CD was not accompanied by decreased cellular TLR responses (51). Similarly, augmentation of NOD2 signaling by treatment of NOD2-intact mice with exogenously administered MDP led to prevention of colitis induced by known colitis-inducing agents such as DSS or TNBS, whereas such treatment did not prevent colitis in NOD2-deficient mice (50). Finally, in studies in which the effect of administration of MDP was taken one step further, treatment with exogenous MDP prevented colitis in NOD2-deficient mice that had been NOD2-repleted by transfection of gut cells with a NOD2-expressing plasmid, but did not prevent colitis in NOD2-deficient mice that had been NOD2-repleted by transfection of gut cells with NOD2-expressing plasmid bearing the frameshift polymorphism (50). Thus, this body of data provided strong evidence suggesting that vigorous or chronic MDP stimulation of intact NOD2 has the effect of downregulating TLR responses by lamina propria cells and leads to amelioration of

colitis; in contrast, the same stimulation of defective NOD2 bearing a CD polymorphism has no such downregulatory effect and does not prevent colitis.

In further and complementary studies, NOD2-deficient mice administered T cells expressing an OVA peptide-specific TCR were found to develop colitis upon rectal administration of *E. coli* organisms engineered to express OVA peptide (ECOVA organisms), but not *E. coli* engineered to express an irrelevant antigen, whereas NOD2-intact mice did not develop colitis under either regimen. In addition, the colitis did not develop in NOD2-deficient mice that were also TLR2-deficient (52). This study thus suggests that a sub-colitogenic immune response to a colonic organism can induce colitis in a NOD2-deficient environment because the response to the organism initiates a colitogenic TLR response that would ordinarily be downregulated by NOD2 stimulation (53). Thus, again, NOD2 deficiency is implicated as a cause of colitis because it leads to defective control of TLR stimulation.

Yet another study supporting the immunoregulatory function of NOD2 focused on the origin of low-level (sub-inflammatory) Th1 cytokine responses in Peyer's patches that can be shown to occur in mice with NOD2 deficiency in the absence of frank colitis (54). In initial studies, it was found that these responses are dependent on the luminal bacterial microflora (or TLR ligands derived from the flora) that translocate into the patch and then stimulate innate TLR responses at this site. The Th1 response thus initiated fosters further translocation and intestinal permeability by activation of myosin light chain kinase (MLCK), a cytokine-activated kinase that causes increased porosity of the tight junctions. On this basis, the Th1 response can be abrogated by gut sterilization with antibiotic administration or by inhibition of the MLCK and can be enhanced by provision of TLR ligands. In further studies, it was established that stimulation of intestinal tissue from NOD2-intact mice in Ussing chambers with TLR ligands increased permeability, but this increase was inhibited by prestimulation with MDP; in contrast, stimulation of intestinal tissue from NOD2-deficient mice with TLR ligands did not increase permeability above an already increased baseline level and, more importantly, in this case, there was no inhibition by prestimulation with MDP. Thus, these studies are consistent with the view that NOD2 signaling has a negative regulatory effect, in this case, focused on low-level cytokine responses in quiescent mice.

Support for the immunoregulatory function of NOD2 very recently has also come from an infectious disease

model in which it was found that NOD2 responses were found to suppress arthritis and carditis occurring during *Borrelia burgdorferi* infection (55). In this study, it was shown that whereas macrophages from mice with NOD2 deficiency produced less pro-inflammatory cytokines when stimulated with *B. burgdorferi*, including less type 1 interferon and TNF- α , they nevertheless developed more arthritis and carditis than WT mice. Subsequent investigation of this discrepancy showed that it was not attributable to NOD2 effects on IL-10 or β -defensin; however, it was associated with the fact that macrophages of NOD2-deficient mice stimulated with *B. burgdorferi* display strikingly decreased cytokine responses when prestimulated with NOD2 ligand. It was therefore speculated that the influence of NOD2 on *B. burgdorferi* infection was biphasic: whereas the acute response to infection was augmented by NOD2 stimulation, the more persistent (chronic) response was diminished by the tolerogenic effect of NOD2 stimulation.

For the most part, the above studies of NOD2-mediated regulation of innate immune responses were antecedent to important studies showing that while pretreatment of peripheral monocyte-derived macrophages from normal individuals with MDP inhibits subsequent pro-inflammatory responses to NOD2, IL-1 β , TLR2, or TLR4 stimulation, it failed to inhibit subsequent responses of this kind to TLR2 and TLR4 stimulation of similar cells from patients with CD bearing frame-shift LRR polymorphisms (56, 57). In contrast, prestimulation of normal macrophages with TNF- α had no effect on subsequent responses. The cytokines inhibited by prestimulation in normal individuals included TNF- α , IL-8 and IL-1 β . These studies closed the circle of proof needed to establish that an important function of NOD2 signaling, in addition to its host defense function, is its ability to regulate or 'cross-tolerize' innate immune responses. In the gastrointestinal tract with its rich complement of bacterial microflora and thus its vulnerability to excessive responsiveness to innate stimuli, this regulatory function of NOD2 is likely to play a key role in mucosal homeostasis and, by the same token, to play a role causing the excessive responses underlying CD when it is genetically defective.

The molecular basis of NOD2 innate immune regulatory function is now under intense study. In one set of studies, it was postulated that the regulatory function of MDP prestimulation was, to a large extent, mediated by NF- κ B1 (p50)-mediated induction of a negative transcription factor, ATF3, that downregulates promoter activity of multiple cytokines ranging from IL-10 to IL-12 and has a generally negative influence on TLR responses (58). However, the

data set supporting this view was somewhat unconvincing because downregulation of NF- κ B1 and downregulation of ATF3 with appropriate siRNAs led to increased responses of both acute and chronic MDP stimulation that blunted, but did not eliminate, the MDP regulatory effect. To achieve a more complete abrogation of the downregulatory response, the authors had to inhibit IL-10, TGF- β , and IRAK-M, an unlikely mix of inhibitory mediators in physiologic settings.

A more promising explanation of NOD2 regulatory function was first suggested by the fact that MDP stimulation of NOD2 upregulates expression of IRF4 a multifunctional factor previously shown to be important in B-cell differentiation, T-cell cytokine production, and several dendritic cell functions (50). The possibility that IRF4 might be involved in NOD2-mediated immunoregulation was initially suggested by the fact that induction of this factor by TLR stimulation was shown to downregulate TLR responses by competing with IRF-5 for binding to MyD88 and thereby disrupting the TLR signaling pathway (59). However, one difference between NOD2 and LPS induction of IRF4 is that activated RICK generated by NOD2 activation binds to IRF4, whereas activated RICK generated by LPS activation does not so bind (50). These data suggesting that IRF4 arising from NOD2 stimulation may have unique regulatory function was then supported by key studies showing that the ability of exogenous MDP administration to protect mice from the development of DSS-colitis was not observed in IRF4-deficient mice (52).

Further insights into the mechanism by which IRF-4 mediates NOD2 innate immune regulation came from studies of binding patterns of NOD2 down-stream activation components (60). Here, it was shown that RICK generated by NOD2 prestimulation of bone marrow dendritic cells of humans binds to various down-stream signaling components including TRAF6, MyD88 and, most notably, to IRF4; at the same time, as noted above, it inhibits NF- κ B activation. In further studies, the significance of such binding became apparent, in that it was shown that RICK overexpressed in HEK293 cells undergoes K63-linked polyubiquitination and such polyubiquitination is inhibited in cells in which IRF4 is also overexpressed; similarly, overexpressed TRAF6 and MyD88 K63-linked polyubiquitination is also inhibited by overexpressed IRF4. These findings were also observed in bone marrow-derived human dendritic cells prestimulated with MDP wherein it was shown that prestimulation induced K63-polyubiquitinated RICK, TRAF6 and MyD88 only when cells were treated with IRF4 siRNA and only cells so treated activated NF- κ B. Taken together, these results showed that MDP prestimulation inhibits subsequent TLR

responses because it induces IRF4 and the latter prevents ubiquitination of down-stream signaling that results in NF- κ B activation.

In vivo confirmation of the above in vitro studies was obtained in two ways. First, mice challenged with TNBS per rectum to induce TNBS-colitis were administered MDP and shown as above to be protected from colitis. Such mice were shown to express greatly increased amounts of IRF-4 in colonic tissue as compared with mice not administered MDP and such IRF-4 was shown to be bound to RICK, TRAF6 and MyD88. In addition, these factors exhibited little, if any, K63-linked polyubiquitination, whereas the same factors in mice not administered MDP exhibited such polyubiquitination. Thus, in vivo stimulation of NOD2 with MDP was shown to have a similar effect on down-stream signaling factors as in vitro prestimulation with MDP. Second, mice challenged with TNBS to induce colitis were administered a tagged plasmid expressing IRF4 in a viral envelop to facilitate cellular entry. The exogenous IRF4 was then shown to bind to RICK and TRAF6 and at the same time to inhibit NF- κ B/MAPK activation as well as pro-inflammatory cytokine production. Thus, administration of IRF4 was shown to by-pass the need to stimulate NOD2 with MDP to achieve a similar anti-inflammatory effect. Overall, we can conclude from these studies that NOD2 signaling exerts a powerful downregulatory effect on TLR signaling in the gut by inhibiting activating polyubiquitination events. By extension therefore, lack of such downregulation due to disrupted NOD2 function as in those individuals with loss-of-function polymorphisms will exhibit increased TLR responses that can lead to CD.

Summary

Studies in search of an explanation for how NOD2 risk polymorphisms and resultant NOD2 dysfunction increase susceptibility to CD have led to two major theories of NOD2-related pathogenesis based on different aspects of NOD2 function and CD mechanisms of disease. One theory is based on well-established evidence that NOD2 has important host defense function and that deficient NOD2 function in mice can lead to infection of the terminal ileum. It has thus focused extensively on the role of NOD2 in Paneth cell α -defensin production and on the clinical fact that NOD2 risk polymorphisms tend to occur in CD patients with inflammation of the terminal ileum, the location of most Paneth cells. However, as discussed in the above review, this theory is weakened by serious concerns that α -defensin

deficiency in CD patients with risk polymorphisms occurs only in those with one of the three possible polymorphisms and appears to be a secondary phenomenon resulting from the pre-existing inflammation. In addition, whereas NOD2 dysfunction is fairly clearly a cause of increased susceptibility to intestinal infection in mice, this association does not seem to hold for humans as there is little evidence that risk polymorphisms lead to intestinal infection either in CD or in normal individuals with such polymorphisms. Another observation related to NOD2 function relating to 'control' of gut microbiota is that there is now evidence that NOD2 deficiency, at least in mouse models, can lead to changes in the intestinal microbiome that have the capacity to intensify a pre-existent inflammation. Thus, it now appears that even if NOD2 dysfunction does not lead to immunodeficiency and the appearance of gut pathogens, it can lead to a microbiome with enhanced colitogenic capacity.

A second theory of NOD2-based CD pathogenesis is based on the idea that the host defensive function of NOD2 is rapidly superseded by a immunoregulatory function that applies to TLR-mediated innate response in general and to

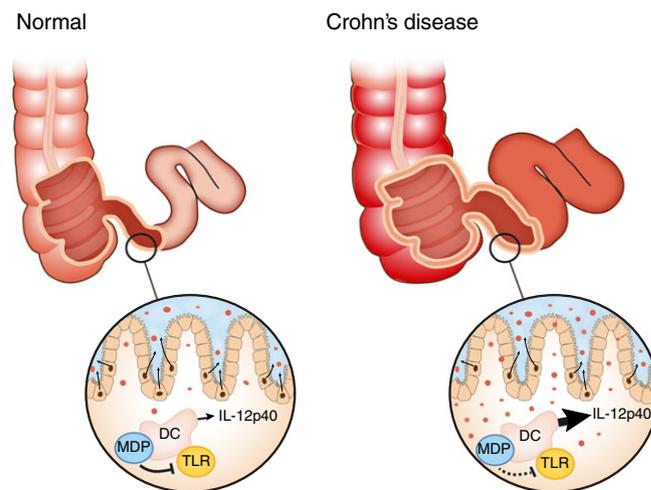


Fig. 1. NOD2 dysfunction and increased risk of Crohn's inflammation. In the normal terminal ileum and adjacent ascending colon (left panel), NOD2 function maintains a gut microbiome with limited colitogenic potential due to epithelial α -defensin production and/or other host defense functions mediated by macrophages and dendritic cells (black dots in epithelial cells represent α -defensins produced by crypt Paneth cells). MDP stimulation of NOD2 associated with dendritic cell (DC) inhibits excessive innate TLR responses and gut homeostasis is maintained. In the inflamed terminal ileum and colon (right panel), there is increased entry of bacteria or bacterial products (red dots) into the lamina propria possibly due to an altered gut microbiome; this leads to increased TLR stimulation of macrophages and dendritic cells that is not well regulated by NOD2; excessive pro-inflammatory cytokine production (IL-12) and loss of gut homeostasis is the result.

intestinal TLR responses in particular. The most dramatic and incontrovertible observation supporting the reality of this response is the ability of exogenously administered NOD2 ligand to prevent or even treat experimental colitis. The molecular basis of this immunoregulatory phenomenon is gradually emerging and appears to be due to the ability of NOD2 stimulation to activate negative down-stream signaling that inhibits pro-inflammatory responses.

These theories of NOD2 disease pathogenesis are not mutually exclusive. It seems possible that the enhanced colitogenic potential of the microbiome associated with a NOD2 defect mentioned above could actually be due to its capacity to subject the mucosal immune system to increased exposure to organisms or their products and thus fuel inflammation caused by a basic increase in the responsiveness of the innate immune system (Fig. 1).

References

1. Jostins L, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012;**491**:119–124.
2. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nature Rev Immunol* 2006;**6**:9–20.
3. Hugot J-P, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;**411**:599–603.
4. Lala S et al. Crohn's disease and the NOD2 gene: a role for Paneth cells. *Gastroenterology* 2003;**125**:47–57.
5. Vaishnava S, Behrendt C, Ismail A, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *PNAS* 2008;**105**:20858–20863.
6. Kobayashi KS, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005;**307**:731–734.
7. Hermiston ML, Gordon JI. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science* 1995;**270**:1203–1207.
8. Petnicki-Ocwieja T, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci USA* 2009;**106**:15813–15818.
9. Biswas A, Petnicki-Ocwieja T, Kobayashi KS. Nod2: a key regulator linking microbiota to intestinal mucosal immunity. *J Mol Med (Berl)* 2012;**90**:15–24.
10. Biswas A, et al. Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum. *Proc Natl Acad Sci USA* 2010;**107**:14739–14744.
11. Wehkamp J, et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α -defensin expression. *Gut* 2004;**53**:1658–1664.
12. Wehkamp J, et al. Reduced Paneth cell α -defensins in ileal Crohn's disease. *Proc Natl Acad Sci USA* 2005;**102**:18129–18134.
13. Simms LA, Doecke JD, Walsh MD, Huang N, Fowler EV, Radford-Smith GL. Reduced α -defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut* 2008;**57**:903–910.
14. Shanahan MT, et al. Mouse Paneth cell antimicrobial function is independent of NOD2. *Gut* 2014;**63**:903–910.
15. Garabedian EM, Roberts LJ, McNeven MS, Gordon JI. Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice. *J Biol Chem* 1997;**272**:23729–23740.
16. Salzman NH, et al. Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol* 2010;**11**:76–83.
17. Raetz M, et al. Parasite-induced TH1 cells and intestinal dysbiosis cooperate in IFN- γ -dependent elimination of Paneth cells. *Nat Immunol* 2013;**14**:136–142.
18. Rehman A, et al. Nod2 is essential for temporal development of intestinal microbial communities. *Gut* 2011;**60**:1354–1362.
19. Mondot S, et al. Altered gut microbiota composition in immune-impaired Nod2^{-/-} mice. *Gut* 2012;**61**:634–635.
20. Smith P, et al. Host genetics and environmental factors regulate ecological succession of the mouse colon tissue-associated microbiota. *PLoS One* 2012;**7**:e30273.
21. Couturier-Maillard A, et al. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *J Clin Invest* 2013;**123**:700–711.
22. Garrett WS, et al. Colitis associated colorectal cancer driven by T-bet deficiency in dendritic cells. *Cancer Cell* 2009;**16**:208–219.
23. Garrett WS, et al. Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. *Cell Host Microbe* 2010;**8**:292–300.
24. Frank DN, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel disease. *Inflamm Bowel Dis* 2011;**17**:1–12.
25. Amendola A, Butera A, Sanchez M, Strober W, Boirivant M. Nod2 deficiency is associated with an increased mucosal immunoregulatory response to commensal microorganisms. *Mucosal Immunol* 2008;**1**:391–404.
26. Frank DN, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. *Proc Natl Acad Sci USA* 2011;**17**:179–184.
27. Sokol H, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008;**105**:16731–16736.
28. Vanderploeg R, Panaccione R, Ghosh S, Rioux K. Influence of intestinal bacteria in human inflammatory bowel disease. *Infect Dis Clin N Am* 2010;**24**:977–993.
29. Gophna U, Sommerfeld K, Gophna S, Doolittle WF, Veldhuyzen van Zanten SJ. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *J Clin Microbiol* 2006;**44**:4136–4141.
30. Baumgart M, et al. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escheria coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the leum. *ISME J* 2007;**1**:403–418.
31. Willing B, et al. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm Bowel Dis* 2009;**15**:653–660.
32. Sokol H, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis* 2009;**15**:1183–1189.
33. Swidsinski A, Loening-Baucke V, Vanechoutte M, Doerffel Y. Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. *Inflamm Bowel Dis* 2008;**14**:147–161.
34. Schwartz A, Jacobi M, Frick JS, Richter M, Rusch K, Köhler H. Microbiota in pediatric inflammatory bowel disease. *J Pediatr* 2010;**157**:240–244.
35. Atarashi K, et al. Treg inductions by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 2013;**500**:232–238.
36. Round JL, Mazmanian SK. Inducible FoxO3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci USA* 2010;**107**:12204–12209.
37. Round JL, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 2011;**332**:974–977.
38. Swidsinski A, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* 2005;**43**:3380–3389.
39. Darfeuille-Michaud A, et al. High prevalence of adherent-invasive Escheria coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004;**127**:412–421.
40. Barnich N, et al. CEACAM6 acts as a receptor for adherent-invasive E coli, supporting ileal mucosa colonization in Crohn disease. *J Clin Invest* 2007;**117**:1566–1574.
41. Chaissaing B, et al. Crohn disease-associated adherent-invasive E. coli bacteria target mouse and

- human Peyer's patches via long polar fimbriae. *J Clin Invest* 2011;**121**:966–975.
42. Glasser AL, Boudeau J, Barnich N, Perruchot MH, Colombel JF, Darfeuille-Michaud A. Adherent invasive *Escherichia coli* strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. *Infect Immun* 2001;**69**:5529–5537.
 43. Bringer MA, Billard E, Glasser AL, Colombel JF, Darfeuille-Michaud A. Replication of Crohn's disease-associated AIEC within macrophages is dependent on TNF- α secretion. *Lab Invest* 2012;**92**:411–419.
 44. Drouet M, et al. AIEC colonization and pathogenicity: influence of previous antibiotic treatment and preexisting inflammation. *Inflamm Bowel Dis* 2012;**18**:1923–1931.
 45. Elinav E, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 2011;**145**:745–757.
 46. Fritz JH, et al. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol* 2005;**35**:2459–2470.
 47. Travassos LH, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* 2010;**11**:55–62.
 48. Cooney R, et al. A NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* 2010;**16**:90–97.
 49. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 2004;**5**:800–808.
 50. Watanabe T, et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J Clin Invest* 2008;**118**:545–559.
 51. Yang Z, et al. NOD2 transgenic mice exhibit enhanced MDP-mediated down-regulation of TLR2 responses and resistance to colitis induction. *Gastroenterology* 2007;**133**:1510–1521.
 52. Watanabe T, Kitani A, Murray PJ, Wakatsuki Y, Fuss IJ, Strober W. Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. *Immunity* 2006;**25**:473–485.
 53. Strober W, Fuss IJ, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007;**117**:514–521.
 54. Barreau F, et al. Nod2 regulates the host response towards microflora by modulating T cell function and epithelial permeability in mouse Peyer's patches. *Gut* 2010;**59**:207–217.
 55. Petnicki-Ocwieja T, et al. NOD2 suppresses *Borrelia burgdorferi* mediated murine Lyme arthritis and carditis through induction of tolerance. *PLoS One* 2011;**6**:e17414.
 56. Hedl M, Li J, Cho JH, Abraham C. Chronic stimulation of Nod2 mediates tolerance to bacterial products. *Proc Natl Acad Sci USA* 2007;**104**:19440–19445.
 57. Hedl M, Abraham C. Secretory Mediators regulate Nod2-induced tolerance in human macrophages. *Gastroenterology* 2010;**140**:231–241.
 58. Zheng S, Abraham C. NF- κ B1 inhibits NOD2-induced cytokine secretion through ATF3-dependent mechanisms. *Mol Cell Biol* 2013;**33**:4857–4871.
 59. Negishi H, et al. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci USA* 2005;**102**:15989–15994.
 60. Watanabe T, et al. NOD2 downregulates colonic inflammation by IRF4-mediated inhibition of K63-linked polyubiquitination of RICK and TRAF6. *Mucosal Immunol*. doi:10.1038/mi.2014.19. [Epub ahead of print].